Reduction of Activity, but no Decrease in Concentration, of Erythrocyte Cu,Zn-superoxide Dismutase by Hyperglycaemia in Diabetic Patients

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Cu,Zn-superoxide dismutase(SOD) activity in erythrocytes is affected by various diseases, including diabetes mellitus (DM). We investigated changes in the Cu,Zn-SOD activity compared to changes in the Cu,Zn-SOD concentration in erythrocytes obtained from patients with Type 2 (non-insulin-dependent) diabetes mellitus. Cu,Zn-SOD activity in erythrocytes was significantly lower in Type 2 DM patients than in healthy non-diabetic subjects. The activity correlated negatively with HbA_{1c}, but not with other indicators of metabolic control, such as fasting blood glucose or plasma cholesterol or triglyceride. However, there was no statistically significant difference in erythrocyte concentration of Cu,Zn-SOD between diabetic and control samples. Concentration did not correlate with enzymatic activity or HbA_{1c}. These findings indicate that the inactivation of Cu,Zn-SOD activity in erythrocytes of Type 2 DM patients by hyperglycaemia may be slow, because there is a negative correlation between the enzyme activities and HbA_{1c} levels, but not fasting blood glucose levels. This is consistent with glycosylation of the active site of Cu,Zn-SOD, without any effect of hyperglycaemia on the concentration of Cu,Zn-SOD.

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Introduction

Excessive production and activity of reactive oxygen species are harmful to tissues. Superoxide dismutase (SOD) catalyses the conversion of the superoxide anion to oxygen and hydrogen peroxide (H_2O_2) and is considered to protect tissue from oxidative damage. Recently, a negative correlation was reported between changes in Cu,Zn-SOD activity in erythrocytes and plasma glucose levels in Type 2 (non-insulin-dependent) diabetic patients with diabetic complications.[1,2] The observed decrease in Cu,Zn-SOD activity is due to glycosylation of the active site of the enzyme.[3] Based on these findings, we investigated whether hyperglycaemia *per se* might influence Cu,Zn-SOD activity and its concentration in erythrocytes from patients with Type 2 (non-insulin-dependent) diabetes mellitus (DM).

Materials and Methods

The subjects were 17 Type 2 DM (11 female) inpatients at our University Hospital (Table 1). Their ages ranged from 25 to 71 years, with a mean (± SD) known duration of diabetes of 10.0 ± 8.3 years. One was treated by diet alone, 11 were taking oral hypoglycaemic agents (OHA) and 5 were using insulin. Ten healthy non-diabetic volunteers (aged 22 to 40 years, 5 male) were also recruited. After an overnight fast, blood samples were obtained from an antecubital vein. Erythrocytes were separated by centrifugation at 700 x*g* for 10 min at 4 °C. The cells were washed twice with saline and the erythrocyte suspension was adjusted to 10^5~10^6 cells μl^{-1}. Fifty microlitres were mixed and shaken with 2.0 ml saline and 0.5 ml ethanol: chloroform (5:3) solution, and then centrifuged at 500 x*g* for 20 min at 4 °C to precipitate haemoglobin, which affects SOD activity. Fifty microlitres

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Table 1. Type 2 DM patients studied

Case number	Sex	Age (yr)	Duration of DM (yr)	Treatment	Smoking habit	Intake of vitamin E	FBG (mmol l^{-1})	HbA_{1c} (%)	SOD activity ($\times 10^2$ μg gHb^{-1})	Complication		
										Retino	Neuro	Nephro
1	M	45	5.0	glibenclamide	+	–	5.1	10.4	3.11	–	–	–
2	F	49	13.0	glibenclamide	–	+	8.2	11.6	3.81	+	–	+
3	M	31	0.4	glibenclamide	+	–	7.8	12.1	3.53	–	–	–
4	F	63	10.0	glibenclamide	+	+	10.9	10.6	4.47	–	+	–
5	F	71	10.0	gliclazide	–	–	12.2	13.1	3.22	–	+	–
6	F	51	26.0	insulin	–	–	32.0	13.1	3.60	+	+	+
7	F	51	7.0	glibenclamide	–	+	10.5	9.9	4.73	+	–	+
8	F	57	0.3	gliclazide	–	–	10.5	11.5	4.69	–	–	+
9	F	71	10.0	gliclazide	–	–	7.8	10.3	4.10	–	+	–
10	F	25	1.0	gliclazide	–	–	5.2	6.5	4.57	–	–	–
11	M	50	22.0	insulin	+	–	26.5	14.0	2.76	+	–	+
12	F	71	20.0	insulin	–	–	7.3	8.0	3.44	+	+	–
13	M	42	2.0	diet	–	–	14.9	13.4	3.05	–	–	–
14	M	59	0.5	gliclazide	–	–	10.2	14.0	3.95	–	–	–
15	F	61	20.0	glibenclamide	–	+	9.3	10.0	3.68	+	+	–
16	M	57	8.0	insulin	+	+	7.3	5.9	4.16	+	+	+
17	F	59	15.0	insulin	–	–	12.8	13.3	3.40	+	+	–

FBG, fasting blood glucose; retino, retinopathy; neuro, neuropathy; nephro, nephropathy.

of the aqueous supernatant were used for Cu,Zn-SOD assay. The Cu,Zn-SOD activity was proportional to the amount of the supernatant.

Cu,Zn-SOD activity was measured by the xanthine oxidase(XOD)-NH_2OH method.[4,5] The concentration of Cu,Zn-SOD was determined using the highly sensitive enzyme immunoassay method provided by Kuroda *et al*.[6] The coefficients of variation for the intra- and inter-assays were 2.2 % and 3 % for SOD activity and below 14 % for SOD concentration, respectively.

Because of the possibility that treatment modality might affect SOD activity, we examined the effect of insulin and OHA (gliclazide and glibenclamide) on the assay system of the SOD activity *in vitro*. This assay system includes two enzyme reactions, namely xanthine oxidase (XOD) and SOD reactions. The addition of 0.6, 1.2, 3.0, and 6.0×10^4 μmol l^{-1} gliclazide to the reaction mixture containing a constant SOD concentration showed optical densities of 0.496, 0.505, 0.490, and 0.489 measured at 550 nm. These differences in optical density in the presence of the different drug concentrations were not statistically significant. The effect of gliclazide on SOD activity was similar to that for XOD. Glibenclamide was similarly without effect on enzyme activity *in vitro*.

We also examined the effect of human recombinant insulin on SOD activity. Commercially available human insulin (Eli Lilly and Company, Indianapolis, Ind., USA) contains stabilizers (glycerin 16 mg ml^{-1} and metacresol 2.5 mg ml^{-1}) which affect SOD activity. Therefore, the stabilizers were removed by using PD-10 column chromatography. The purified human insulin did not influence either XOD or SOD activities in concentrations of 1.12–21.9 U l^{-1}.

The plasma glucose concentration was measured by the glucose oxidase method. Glycated haemoglobin (HbA_{1c}) concentration was measured by HPLC.[7] Plasma lipids (triglyceride,[8] cholesterol,[9] and lipoperoxide[10]) were determined by standard methods. Statistical analysis was performed by ANOVA for unpaired values and Spearman correlation test.

Results

The blood glucose levels were 11.9 ± 1.7 mmol l^{-1} for diabetic patients and 5.2 ± 0.3 for healthy non-diabetic controls. HbA_{1c} concentrations were 11.04 ± 2.48 for diabetic patients and 5.22 ± 0.43 for healthy non-diabetic subjects.

Cu,Zn-SOD activity in erythrocytes was significantly decreased in diabetic patients compared with that in healthy non-diabetic subjects (Table 2). There was a significant negative correlation between Cu,Zn-SOD activities and HbA_{1c}. However, there was no correlation between Cu,Zn-SOD and fasting blood glucose levels (Figure 1) or lipid concentrations (Figure 2).

In contrast, Cu,Zn-SOD concentrations in erythrocytes of Type 2 DM patients were similar to those in the healthy subjects (Table 2). There were no significant correlations between the concentrations of Cu,Zn-SOD and fasting blood glucose levels, HbA_{1c} levels or lipid concentrations in Type 2 DM patients. In addition, there was no correlation between the activities and concentrations of Cu,Zn-SOD in the erythrocytes of Type 2 DM patients.

Discussion

We have demonstrated that the activity of Cu,Zn-SOD, which protects against superoxide radicals, was significantly lower in erythrocytes from diabetic patients

Table 2. Cu,Zn-SOD activities and concentrations in erythrocytes from healthy subjects and NIDDM patients

	Healthy subjects ($n = 10$)	NIDDM patients ($n = 17$)
Cu,Zn-SOD activity		
SOD/RBC ($\times 10^{-9}$ μg/RBC)	13.68 ± 0.68	11.23 ± 0.36[a]
SOD/Hb ($\times 10^{2}$ μg gHb^{-1})	4.82 ± 0.32	3.78 ± 0.15[b]
Cu,Zn-SOD concentration ($\times 10^{2}$ μg gHb^{-1})	5.20 ± 0.82	7.14 ± 0.83

Results expressed as the mean ± SE. RBC, red blood cells; Hb, haemoglobin.
[a]$p < 0.05$ and [b]$p < 0.01$, statistically significant, compared with healthy subjects.

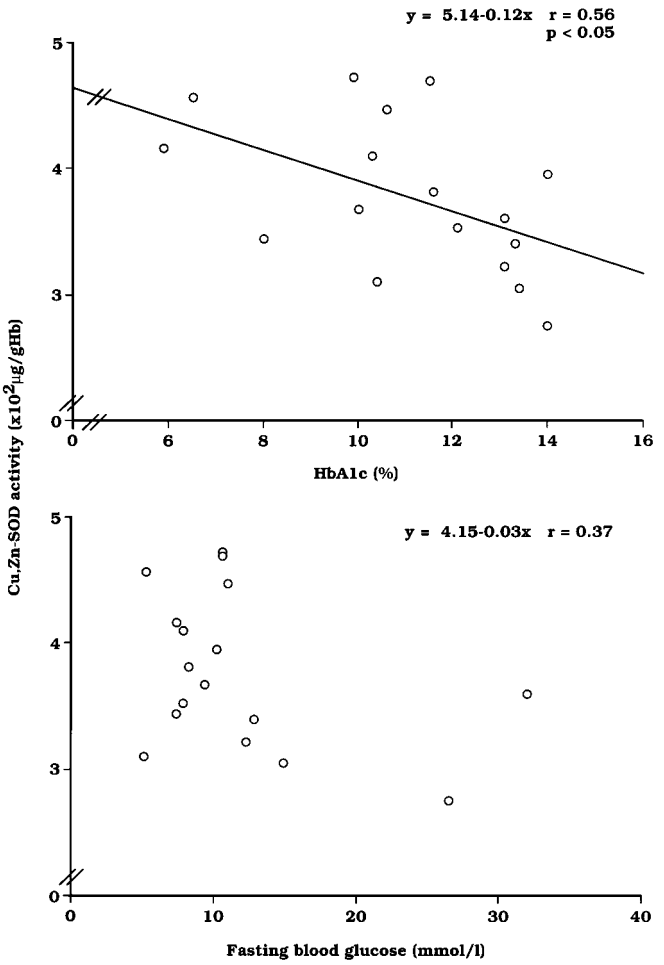

Figure 1. Correlations between Cu,Zn-SOD activities and HbA_{1c} levels or fasting blood glucose (FBG) levels in erythrocytes from Type 2 DM patients

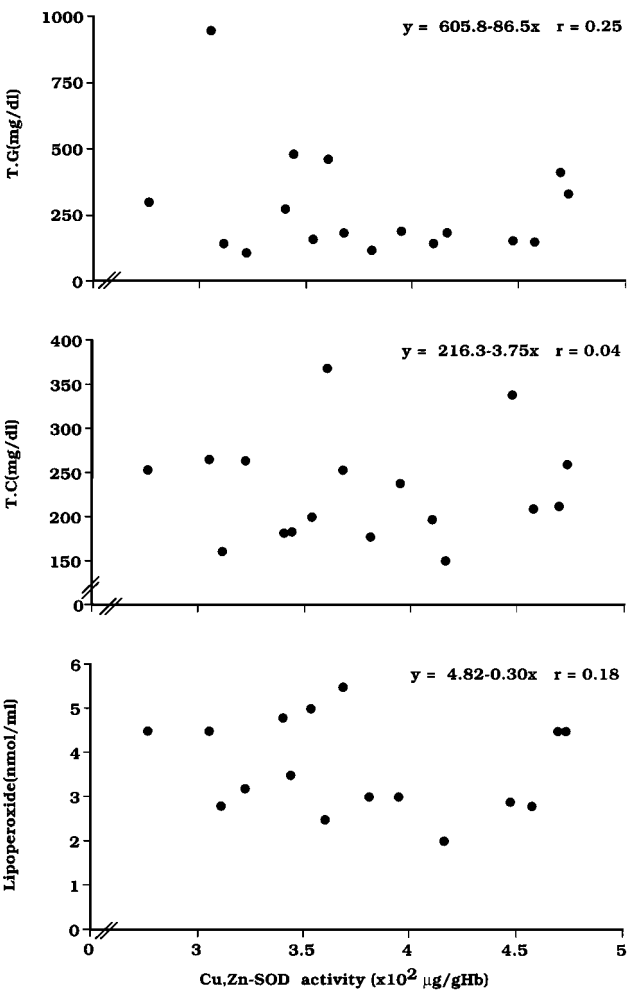

Figure 2. Correlations between Cu,Zn-SOD activities and blood lipid concentrations in Type 2 DM patients. TC, total cholesterol; TG, triglyceride

than in those from healthy subjects, as suggested by previous studies.[11–13] However, we found that the concentrations of Cu,Zn-SOD measured by highly sensitive EIA prepared by Kuroda *et al.*[6] was normal among diabetic patients and did not correlate with Cu,Zn-SOD activity. These findings are consistent with the previous report that Cu,Zu-SOD is inactivated by glycation of the active site of the enzyme, particularly Lys^{122} and Lys^{128}, which are located in the active sites in the binding loop region.[3]

Cu,Zn-SOD activity in erythrocytes in diabetic patients correlated negatively with HbA_{1c}. HbA_{1c} reflects the mean blood glucose level of the preceding 1 to 2 months. Cu,Zn-SOD activity showed no correlation with other indicators, i.e. fasting blood glucose and serum lipid concentrations. In contrast, Oda *et al.*[1] reported that the Cu,Zn-SOD activity in erythrocytes from diabetic patients correlated well with fasting blood glucose levels but not with HbA_{1c} levels.

Fasting blood glucose levels reflect acute changes in diabetic control, but the HbA_{1c} concentration is a marker

of long-term diabetic control. This may explain why Cu,Zn-SOD activity correlated well with HbA_{1c} concentrations, but not with fasting blood glucose levels. Previous studies[1,14,15] report a significant decrease in Cu,Zn-SOD activity in bovine or purified human erythrocytes after 3 days' incubation in high glucose concentrations due to *in vitro* glycosylation. The other studies *in vivo*[2,3] did not present any measurements of SOD concentration, as distinct from SOD activity, in Type 2 DM patients but in our study, SOD concentrations[6] were not affected by diabetes. These data are compatible with a low glycosylation of the active site of Cu,Zn-SOD in erythrocytes in diabetic subjects, resulting in decreased activity, but normal concentrations of the enzyme. It is possible that different treatment modalities for diabetes might influence SOD activity. This is unlikely to have confounded our data however. Christ *et al*. reported that the maximum blood level of glibenclamide after oral administration was 0.2 μmol l^{-1},[16] at which concentrations it is unlikely to affect XOD and SOD activities. Jennings *et al*. also indicated that SOD activity in plasma was increased, rather than inhibited, by gliclazide.[17]

Sundaram *et al*.[2] reported a decrease in SOD activity in erythrocytes obtained from 467 cases of Type 2 DM patients and showed a negative correlation between SOD activity and the duration of the disease. However, in our study the reduction in activity of SOD in the erythrocyte did not clearly parallel the development of DM and its complications. Further studies are necessary to clarify whether alterations in Cu,Zn-SOD activity and the free radical level affect the progress of diabetic complications.

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